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# Single nucleotide primer extension to detect genetic diseases: Experimental application to hemophilia B (factor IX) and cystic fibrosis genes

**(polymerase chain reaction/restriction fragment length polymorphism/denaturing polyacrylamide gel electrophoresis/sickle cell mutation/*ras* oncogenes)**

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**ABSTRACT** **In this report, we describe an approach to detect the presence of abnormal alleles in those genetic diseases in which frequency of occurrence of the same mutation is high (e.g., cystic fibrosis and sickle cell disease), and in others in which multiple mutations cause the disease and the sequence variation in an affected member of a given family is known (e.g., hemophilia B). Initially, from each subject, the DNA fragment containing the putative mutation site is amplified by the polymerase chain reaction. For each fragment two reaction mixtures are then prepared. Each contains the amplified fragment, a primer (18-mer or longer) whose sequence is identical to the coding sequence of the normal gene immediately flanking the 5′ end of the mutation site, and either an $\alpha$-$^{32}$P-labeled nucleotide corresponding to the normal coding sequence at the mutation site or an $\alpha$-$^{32}$P-labeled nucleotide corresponding to the mutant sequence. Single nucleotide primer extensions are then carried out and analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography. As predicted by the Watson–Crick base-pair rule, in the wild type only the normal base, in an affected member only the mutant base, and in carriers both the normal and the mutant base are incorporated into the primer. Thus, an essential feature of the present methodology is that the base immediately 3′ to the template-bound primer is one of those altered in the mutant, since in this way an extension of the primer by a single base will give an extended molecule characteristic of either the mutant or the wild type. The method is rapid and should be useful in carrier detection and prenatal diagnosis of every genetic disease with a known sequence variation.**

One goal of molecular biology is to identify the mutations that cause genetic diseases and to develop strategies and related technologies to diagnose them. Toward this end, in the past decade or so many methodological advances have been made to detect the human genetic abnormalities at the DNA level. These include indirect methods such as linkage analysis by the Southern blotting technique (1) in which the inheritance of a disorder is associated with the presence of a restriction fragment length polymorphism (RFLP)—e.g., Duchenne muscular dystrophy (2). Other indirect methods include RNase A cleavage at mismatches in probe RNA–sample DNA duplexes or denaturing gradient gel electrophoresis for mismatches in probe DNA–sample DNA duplexes—e.g., $\beta$-thalassemia (3, 4). The direct methods include detection with the restriction enzymes or with the allele-specific oligonucleotide (ASO) probes—e.g., the sickle cell mutation (5, 6).

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A majority of the above approaches have now been combined with the polymerase chain reaction (PCR) for diagnosis of the sequence variations (7, 8). Initially, the target DNA is amplified by PCR followed by analysis of the sequence variation by ASO hybridization (e.g., the sickle cell mutation; ref. 9), restriction enzyme analysis (e.g., the sickle cell mutation and a hemophilia B mutation; refs. 10 and 11), RNase A cleavage (e.g., $\alpha_1$-antitrypsin gene *Z* mutation; ref. 12), denaturing gradient gel electrophoresis (e.g., hemophilia A mutations; ref. 13), chemical cleavage (e.g., hemophilia B mutations; ref. 14), and ligation of oligonucleotide pairs or ligation amplification (e.g., the sickle cell mutation; refs. 15–17). Recently, an allele-specific PCR (ASPCR) amplification technique to diagnose point mutations has also been introduced (18). Some of the above techniques do not detect all mutations that involve single nucleotides and are technically quite demanding (12–16). Others require optimization of conditions that allows specific hybridization of the ASO probe (9) or specific amplification of the selected allele by ASPCR (19).

We have developed a simple strategy that detects known DNA sequence variations by as little as one nucleotide. The strategy is based on the fidelity of the DNA polymerase, in that it only adds the correctly paired nucleotide onto the 3′ terminus of the template-bound primer. Since only one nucleotide is provided in the extension reaction and it is radiolabeled, one can easily discern the identity of the first nucleotide in the template DNA adjacent to the 3′ end of the primer. The approach is fundamentally different from any of the previously described approaches, and its use is tested here in the detection of hemophilia B and cystic fibrosis (CF) mutations. An initial account of this work pertaining to hemophilia B mutations has been presented in abstract form (20).

## EXPERIMENTAL PROCEDURES

**Materials.** *Thermus aquaticus* DNA polymerase (*Taq* polymerase) was obtained from Cetus. $\alpha$-$^{32}$P-labeled nucleotides (10 $\mu$Ci/$\mu$l; 3000 Ci/mmol; 1 Ci = 37 GBq) were obtained from DuPont-New England Nuclear. Genomic DNA was

Abbreviations: RFLP, restriction fragment length polymorphism; ASO, allele-specific oligonucleotide; PCR, polymerase chain reaction; ASPCR, allele-specific PCR; CF, cystic fibrosis; SNuPE, single nucleotide primer extension; IX:C, factor IX clotting activity; IX:Ag, factor IX antigen level.

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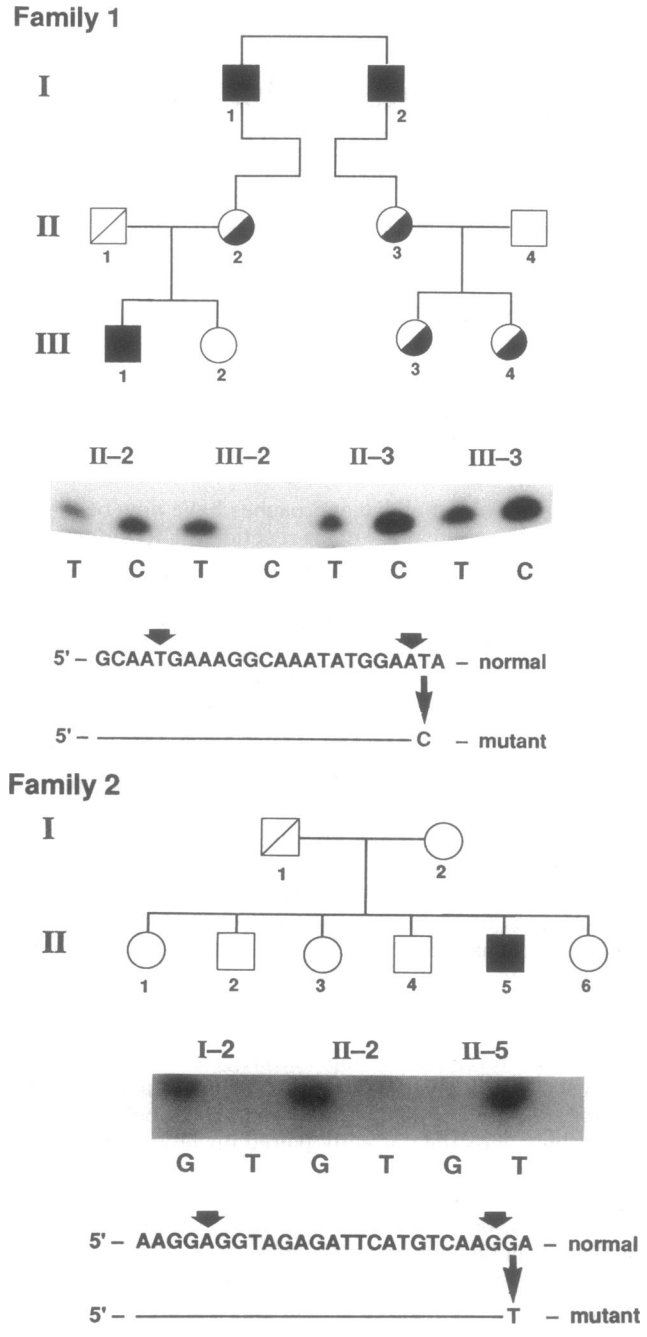


FIG. 1. Hemophilia B carrier detection with SNuPE in two families with point mutations in exon VIII of the factor IX gene. Family 1 has an $Ile^{397} \rightarrow Thr$ (nucleotide 31311 T → C) mutation (32) and family 2 has a $Gly^{363} \rightarrow Val$ (nucleotide 31209 G → T) mutation (33). Initially, exon VIII from each subject was amplified from the genomic DNA by the standard PCR (28, 29) using the two primers corresponding to nucleotides 30760–30780 and 31360–31379 of the factor IX gene (26). The isolated amplified fragments were used for SNuPE reactions. The extension of the SNuPE primer for each reaction was then analyzed by gel electrophoresis and autoradiography. A 5-μl aliquot of each sample was mixed with an equal volume of gel loading buffer (80% formamide/50 mM Tris borate, pH 8.3/1 mM EDTA/0.1% xylene cyanol/0.1% bromophenol blue), heat denatured at 90°C for 1 min, and loaded onto a 6% polyacrylamide gel containing 8 M urea. Gels (17 cm) were run at 300 V for 2 hr to obtain adequate resolution of the extended primer from the free nucleotide. Autoradiographs of gels were made by overlaying Kodak X-AR5 film and exposing for 20–30 min at room temperature. The sequence of SNuPE primer for each family is indicated by the top two arrows. The causative base change in each family is also depicted. For each individual, two SNuPE reactions were carried out; the single radiolabeled nucleotide included in the SNuPE reaction was either T (normal) or C (mutant) for family 1 and was either G (normal) or T (mutant) for family 2. When only the wild-type base was incorporated into the SNuPE primer, the subject was considered normal; when only the mutant base was incorporated into the primer, the subject was considered a hemophiliac; when both bases (one in each reaction) were incorporated into the primer, the subject was considered a carrier of the disease. The autoradiographs depicted in this figure show results from a limited number of subjects. Square, male; circle, female; solid symbol, hemophiliac; open symbol, normal; half-solid symbol, carrier of hemophilia; slashed symbol, deceased.

isolated from the blood leukocytes by standard techniques. Use of volunteer blood donors was approved by the human subjects committees of St. Louis University and the University of Southern California.

**Coagulation Assays.** Factor IX clotting activity (IX:C) was measured as described (21). Factor IX antigen levels (IX:Ag) were determined by an antibody neutralization assay and also by an electroimmunoassay as described (21). Citrated human plasma pooled from 20 healthy donors was defined as containing 100% IX:C (range, 60–140%) and 100% IX:Ag (range, 60–135%).

**Restriction Endonuclease Analyses.** The probes used were DXS99/*Sst* I (22), DXS100/*Taq* I (23), DXS102/*Taq* I (24), DXS105/*Taq* I (24), and Ala35/*Taq* I (25). Probes DXS99, DXS100, and DXS102 detect RFLPs on the telomeric side of the factor IX gene, whereas the probe DXS105 detects RFLPs on the centromeric side of the factor IX gene. The probe Ala35 is an intragenic probe and is located near the 5′ end of intron D of the factor IX gene (25, 26). Since this marker is within the factor IX gene, it has a very low risk of recombination. Also, as yet no recombination has been observed between the DXS99 marker and the factor IX gene (22). The DXS100 marker is at a 9% recombination distance (23), the DXS102 marker is at a 2% recombination distance (24), and the DXS105 marker is at an 8% recombination distance (24) from the factor IX gene. Restriction enzyme cleavage, transblotting, preparation of $^{32}P$-labeled probes, hybridization, and autoradiography were performed according to established procedures (1, 27).

**PCR Amplification and Isolation of the Amplified DNA.** The set of primers used for PCR amplification of exon VIII corresponding to nucleotides 30760–30780 and 31360–31379 of the factor IX gene (26) has been provided earlier from this laboratory (28). Target sequences in the genomic DNA were amplified essentially according to Saiki *et al.* (29). Our procedure has been described in detail in an earlier publication (28). After amplification, the DNA was electrophoresed on 1% agarose gel in Tris acetate/EDTA buffer (27). The segment of the gel containing the amplified region was cut out and mixed with an equal volume of phenol (pH 8.0) and frozen at −70°C for a minimum of 10 min. The sample was thawed at 37°C for 10 min and briefly centrifuged in an Eppendorf tube. The DNA in the upper aqueous layer was ethanol-precipitated and stored until used. The exon region containing the 1611- to 1708-base-pair (bp) segment (30) of the CF gene containing the most common deletion mutation ($Phe^{508}$) was amplified by using the two PCR primers (C16B and C16D) given earlier by Keram *et al.* (31). After amplification, the DNA was electrophoresed on 6% polyacrylamide gel in Tris borate/EDTA buffer (27). The segment of the gel containing the amplified fragment (≈100 bp) was cut out and the DNA was extracted by electroelution (40 V per 12 hr) using the dialysis membrane tubing (Spectra/Por 2) in Tris borate/EDTA buffer (27). The DNA was ethanol-precipitated and stored until used.

**Single Nucleotide Primer Extension (SNuPE).** Each SNuPE reaction was carried out in a 50-μl vol containing ≈100 ng of the amplified DNA fragment, 1 μM SNuPE primer, 2 units of

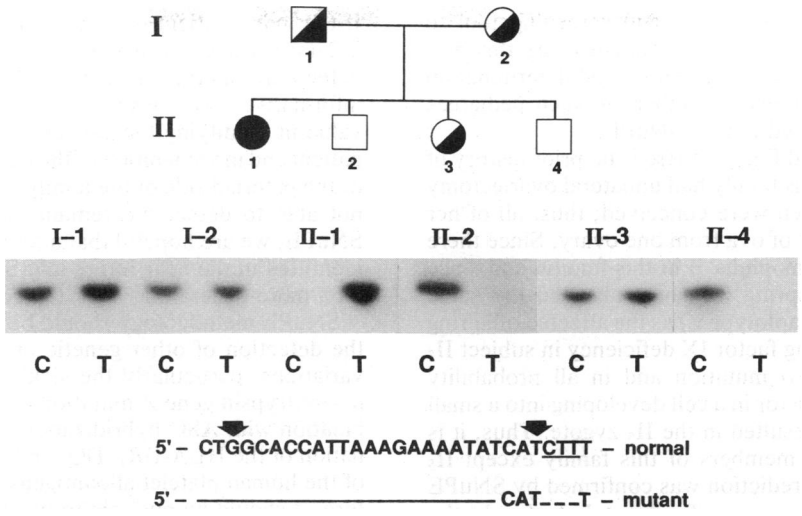


FIG. 2. Detection of the Phe$^{508}$ deletion mutation with SNuPE in a CF family. PCR amplification of the exon region (1611- to 1708-bp cDNA segment) of genomic DNA containing the Phe$^{508}$ deletion mutation and execution of the SNuPE reaction was carried out. Details of the analysis of primer extensions and preparation of the autoradiographs are given in the legend to Fig. 1. The $^{32}$P-labeled single nucleotide included in the SNuPE reaction was either C (normal) or T (mutant). In a fully informative family, such as shown in this figure, when only the C base was incorporated into the primer, the subject was considered normal ($II_2$ and $II_4$); when only the T base was incorporated into the primer, the subject was considered a CF patient ($II_1$); when both C and T were incorporated into the primer, the subject was considered a carrier of the CF gene ($I_1$, $I_2$, and $II_3$). Square, male; circle, female; solid symbol, CF patient; open symbol, normal; half-solid symbol, carrier of CF gene.

*Taq* polymerase, and 1 μl of the appropriate α-$^{32}$P-labeled nucleotide (10 μCi/μl; 3000 Ci/mmol). The buffer used was 10 mM Tris·HCl (pH 8.3) containing 50 mM KCl, 5 mM $MgCl_2$, and 0.001% (wt/vol) gelatin. The samples were subjected to one cycle consisting of a 2-min denaturation period at 95°C, a 2-min annealing period at 60°C, and a 2-min primer-extension period at 72°C. The sequence of the SNuPE primer for each family is given in Figs. 1 and 2. Details of gel electrophoresis and autoradiography for detection of the extended primer are also given in the legends to Figs. 1 and 2.

## RESULTS AND DISCUSSION

Hemophilia B is an X chromosome-linked bleeding disorder caused by the absence of factor IX coagulant activity. We recently described two naturally occurring point mutations in the protease domain of factor IX that lead to impaired macromolecular catalysis by the mutated enzymes (32, 33). In both families, before defining the mutation sites within exon VIII of the factor IX gene, we determined IX:C/IX:Ag ratios, and we performed linked RFLP analysis to establish the carrier status of female members in these pedigrees. In family 1, RFLPs were not very informative because of $II_1$ being deceased and $II_4$ being unavailable (see Table 1 and Fig. 1). Although we could establish that haplotype A ($III_1$) carried the marker for hemophilia B in this family and all three females carried this haplotype A, it was not possible to rule out that a normal haplotype A in these females could have come from the male parents—i.e., $II_1$ or $II_4$. From the IX:C/IX:Ag ratios (close to 0.5 being a carrier, close to 1 being a noncarrier, and close to 0.75 being noninformative), we could not predict the carrier status of $III_2$ and $III_4$ (see Table 1); moreover, the carrier status of $III_3$, predicted not to be a carrier by the IX:Ag/IX:C ratio, turned out to be wrong (see below).

After identification of the causative base change (T → C at nucleotide 31311), we applied SNuPE to determine the carrier status of the females in the above family; $III_2$ was identified a noncarrier and $III_3$ and $III_4$ were identified as carriers of the disease (Fig. 1 and Table 1). From SNuPE analysis, we could also determine that subject $III_2$ had inherited the normal haplotype A from the father and that subjects $III_3$ and $III_4$ inherited the haplotype A carrying the mutation from their mother. Since it has been estimated that ≈1 of 5 hemophilia B patients (with factor IX sequence changes) carries the T → C mutation at nucleotide 31311 (34,

Table 1. Genotypes and IX:C and IX:Ag in the two hemophilia B pedigrees

| Patient | IX:C | IX:Ag | IX:C/IX:Ag | Haplotype | Probabilistic carrier status | Carrier status from SNuPE |
|---|---|---|---|---|---|---|
| Family 1 | | | | | | |
| $I_1$, $I_2$, $II_1$* | | | | | | |
| $II_2$ | 115 | 120 | 0.95 | A/B | Obligate C | C |
| $II_3$ | 50 | 122 | 0.42 | A/C | Obligate C | C |
| $II_4$† | | | | | | |
| $III_1$ | <1 | 79 | 0.012 | A | | |
| $III_2$ | 83 | 122 | 0.68 | A/B | ? | NC |
| $III_3$ | 60 | 66 | 0.91 | A/C | NC | C |
| $III_4$ | 51 | 74 | 0.67 | A/C | ? | C |
| Family 2 | | | | | | |
| $I_1$* | | | | | | |
| $I_2$ | 100 | 140 | 0.71 | A/B | ? | NC |
| $II_1$ | 79 | 86 | 0.92 | A/C | NC | NC |
| $II_2$ | 72 | 81 | 0.89 | B | | |
| $II_3$ | 123 | 126 | 0.98 | B/C | NC | NC |
| $II_4$ | 78 | 83 | 0.94 | A | | |
| $II_5$ | <1 | 68 | 0.014 | B | | |
| $II_6$ | 76 | 90 | 0.85 | B/C | NC | NC |

Restriction enzymes used for haplotype analysis of family 1 were DXS99, DXS100, Ala35, and DXS105. Haplotype A is defined as 1/1/1/2, haplotype B is defined as 2/1/2/2, and haplotype C is defined as 2/1/1/2. Restriction enzymes used for haplotype analysis of family 2 were DXS99, DXS100, Ala35, DXS102, and DXS105. Haplotype A is defined as 1/1/1/1/1, haplotype B is defined as 2/2/2/1/2, and haplotype C is defined as 2/1/2/1/2. For both families, number 1 refers to the absence of a restriction site and number 2 refers to its presence. C, carrier; NC, noncarrier; ?, noninformative.

*Deceased.

†Not available.

35), SNuPE could serve as a powerful screening tool to determine whether or not the index patient bears this particular mutation. If so, prenatal diagnosis and determination of the carrier status of female members in such pedigrees could be accurately carried out by SNuPE.

In family 2 (Table 1 and Fig. 1), there is no prior history of bleeding. Subject $I_2$ in this family had unilateral ovariectomy before any of her children were conceived; thus, all of her offspring are the product of ova from one ovary. Since there is no prior history of hemophilia B in this family, and since an unaffected male offspring ($II_2$) has inherited the same maternal chromosome (haplotype B) as the affected offspring ($II_5$), the mutation causing factor IX deficiency in subject $II_5$ is most likely a *de novo* mutation and in all probability occurred in a single ovum (or in a cell developing into a small part of the ovary) that resulted in the $II_5$ zygote. Thus, it is highly probable that all members of this family except $II_5$ would be normal. This prediction was confirmed by SNuPE (Fig. 1 and Table 1). However, if subject $I_2$ had only the offspring $II_4$, $II_5$, and $II_6$ and their haplotypes were known, it would not have been possible to preclude $II_6$ as a carrier. Moreover, if after the diagnosis of hemophilia B in $II_5$, another male fetus was conceived, the diagnosis of hemophilia B could not be made or excluded because, like $II_2$, he might be normal. With a known sequence mutation, the use of SNuPE under these circumstances would obviate such problems.

CF is the most common severe autosomal recessive disorder in the Caucasian population; its clinical, physiologic, and genetic aspects have been reviewed recently (36). The cloning of the CF cDNA has also been described (30), and a 3-bp deletion that removes $Phe^{508}$ from the putative CF protein of 1480 amino acids has been identified as the causative mutation in approximately two-thirds of the CF chromosomes (31, 37). We analyzed 34 chromosomes of 17 normal individuals by the SNuPE method and found that none of them had the 3-bp deletion that results in loss of the amino acid $Phe^{508}$ (Table 2). We also analyzed 74 CF chromosomes (37 CF unrelated patients) and found that 46 of them had the putative 3-bp deletion; this further establishes that indeed ≈60% of the CF chromosomes carry the 3-bp deletion. Using SNuPE, we identified several families homozygous for this mutation. Results from one family are shown in Fig. 2. Each parent has one CF and one normal chromosome and the affected child ($II_1$) has two CF chromosomes, one derived from each parent. Two children ($II_2$ and $II_4$) have inherited the normal chromosome from each parent. Another child ($II_3$) inherited one normal and one CF chromosome and is a carrier of the CF disease.

We now routinely use SNuPE as a screening procedure for determination of the $Phe^{508}$ deletion mutation in CF patients.

Table 2. Prevalence of $Phe^{508}$ deletion mutation in normal and CF chromosomes as detected by SNuPE

| | Total | $Phe^{508}$ deletion | % $Phe^{508}$ deletion |
|---|---|---|---|
| Normal chromosomes | 34 | 0 | 0 |
| CF chromosomes | | | |
| (all unrelated CF patients) | 74 | 46 | 62 |
| 1/1 genotype CF patients | 10 | 0 | — |
| 1/2 genotype CF patients | 36 | 18 | — |
| 2/2 genotype CF patients | 28 | 28 | — |

Haplotype 1 is defined as that in which the CF mutation is at a region other than the $Phe^{508}$ deletion and haplotype 2 is defined as that in which the CF mutation is the $Phe^{508}$ deletion. The predicted numbers of the different genotypes if 62% of the CF chromosomes contained the $Phe^{508}$ deletion mutation are as follows: 10.7 (or $0.38 \times 0.38 \times 74$) for genotype 1/1, 34.9 (or $2 \times 0.38 \times 0.62 \times 74$) for genotype 1/2, and 28.4 (or $0.62 \times 0.62 \times 74$) for genotype 2/2.

If the index patient is fully informative—i.e., has genotype 2/2 (see Table 2)—SNuPE alone can be used for carrier detection and prenatal diagnosis. If the index patient is partly informative—i.e., has genotype 1/2—SNuPE can still be of value in modifying the carrier risk for siblings of the index patient and in recognition of the carriers in either the maternal or the paternal side of the family. Although at present we are not able to detect the remainder of the CF mutations by SNuPE, we are hopeful that additional CF mutations will be identified in the near future and SNuPE may then be useful on a more general basis for CF gene screening.

SNuPE methodology should be immediately applicable to the detection of other genetic diseases of known sequence variations, particularly the sickle cell mutation (9) and the $\alpha_1$-antitrypsin gene *Z* mutation (12). Recently, PCR in combination with ASO hybridization has been used for determination of the HLA-DR, -DQ, and -DP alleles (9, 38, 39), and of the human platelet alloantigens $PI^{A1}$ and $PI^{A2}$ (40). Therefore, it should be possible to use SNuPE for direct analysis of the HLA types and of the platelet alloantigen (C ↔ T) polymorphism. Moreover, SNuPE may also find application in the early detection of codon 12 and codon 61 mutations in *ras* oncogenes, which are estimated to be associated with ≈30% of human tumors (41). Since, compared with ASO hybridization, SNuPE is technically less demanding and takes less time to perform, it may become the method of choice. Recently, ASPCR has been developed in which primers conjugated with fluorescent dyes were used (42). In the future, it may be possible to use the specific nucleotide conjugated with a fluorescent dye in the SNuPE reaction, thus eliminating the need for a radioactive nucleotide.

SNuPE can also be useful in many research settings. For example, in an autosomal genetic disorder, if an investigator finds a mutation (or sequence variation) in a PCR amplified segment of the DNA (obtained from the patient) cloned into PUC18 or M13 vector, he (or she) can readily check, by SNuPE, whether or not both chromosomes carry the same mutation and thus distinguish between the homozygous and the compound heterozygous mutations. In a similar way, if a particular sequence variation is identified in the gene of a patient with an inherited disorder of that gene product, then SNuPE can be used to test the association of the sequence variation with the genetic disease in the pedigree of the family of that patient. The mutations can be verified in the PCR amplified fragments relatively easily by the use of SNuPE, thus eliminating the need for sequencing the fragments in their entirety.

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